Recombinant expression, purification and characterization of Kch, a putative *Escherichia coli* potassium channel protein

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Abstract The *Escherichia coli* gene *kch*, similar in primary structure to eukaryotic voltage-gated potassium channels, was cloned and overexpressed in *E. coli*. The protein was solubilized from the plasma membrane with dodecylmaltopyranoside, lauryldimethylamine oxide or *N*-laurylsarcosine and was purified in milligram amounts by imidazole elution from a nickel-chelate column. The molecular mass of the purified protein in a number of detergents with 12 carbon atom chains suggests that rKch forms primarily tetramers of the 50 kDa monomers. CD spectroscopy of the purified protein indicates a significant α-helical content that is preserved upon addition of SDS.

Key words: Membrane protein; Channel protein; Potassium channel; Kch

1. Introduction

Ion channel proteins, found in essentially all eukaryotes, can be grouped into distinct families and subfamilies [1]. The family of voltage-gated channels shows a common primary topology of four homologous domains or identical subunits. The Ca^{2+} and Na^{+} channels possess these four domains in a single polypeptide, while the K^{+} channels assemble four subunits into a homotetramer to become active [2]. Primary sequence analysis, secondary structure predictions and functional studies of voltage-gated potassium channels indicate that each domain or subunit consists of six transmembrane helices S1–S6 harboring a so called pore region (P-region) between S5 and S6, which is responsible for ion selectivity (for a recent review see [3]).

Recently a putative, K^{+} channel-homologous gene, *kch* (GenBank accession number L12044), has been found in a broad range of *Escherichia coli* K-12 wild type strains [4]. The primary structure shows the presence of six apparent transmembrane regions with a low overall sequence similarity to eukaryotic channels. However, the potassium-specific P-region is 60–75% identical to corresponding regions in various eukaryotic potassium channel proteins [4]. A recent experimental study [5] confirmed the assumed membrane topology with six transmembrane segments and a periplasmatically anchored P segment possibly penetrating between the transmembrane helices. The purpose of this work was to biochemically and biophysically characterize the gene product of *kch* and to provide sufficient material for future electrophysiological characterizations and structural studies.

2. Materials and methods

2.1. Cloning and expression of Kch

E. coli K-12 wild type genomic DNA was purified and used as a template in a PCR (30 cycles at 95°C, 30 s; 63°C, 30 s; 72°C, 1 or 5 min) containing the Vent polymerase and the primers (5′-CTCGGATCCA GTCACTGGGC TACATTCAAA CAAACA-3′) and (5′-GGCTCAAGCT TCTATTTTTG CGCCGATTCT TTACTGTC-3′) at 1 μM. The 1200 bp product and the expression plasmid pET28a(+) were digested with *Bam*HI and *Hin*dIII, gel-purified and ligated with T4 ligase. The resulting clone pET28a(+)-Kch was analyzed by restriction digestion, amplified in DH5α and subjected to DNA sequencing. For protein expression, a 2 ml starter culture from one freshly transformed colony of BL21(DE3) cells was grown overnight and used to inoculate 1.5 l LB medium containing 30 μg/ml kanamycin at 37°C. When the cell density reached $A_{600} = 0.7$, IPTG was added to a final concentration of 0.1 mM and the culture was further grown at 33°C for 3.5 h. After harvesting, the cells were resuspended in 20 ml of buffer containing 50 mM Tris-HCl pH 8.0, 5 mM Na-phosphate, 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT and 3 mM NaN_3 on ice. 0.1 mg/ml lysozyme, 0.12 mg/ml DNase and 0.08 mg/ml RNase were added and the cells were lysed by two runs through a French press. To remove the cell debris (referred to as low centrifugation pellet) the lysate was centrifuged at $10\,000 \times g$ for 30 min at 4°C. The supernatant was centrifuged at 42 000 rpm in a Beckman 45 Ti rotor (about $140\,000 \times g$) for 90 min at 4°C to obtain crude membranes (also referred to as high centrifugation pellet).

2.2. Density gradients

Sucrose gradient centrifugations were carried out with resuspended crude membranes in a Beckman SW41 swinging bucket rotor at 36 500 rpm (about $165\,000 \times g$) at 4°C for more than 18 h according to Osborn and Munson [6]. The mass densities of control sucrose fractions without loaded membrane samples were measured in an optical refractometer. The relative Kch content in the two obtained bands was estimated by running SDS-PAGE, and scanning and integrating the corresponding Coomassie-stained bands. Lipids were separated on silica plated by TLC with a 22:12:2 (v:v:v) chloroform:methanol:ammonium hydroxide mixture as solvent. After drying the plates were stained with ninhydrin, molybdenum blue and charred.

2.3. Solubilization and purification

Crude membranes were resuspended and homogenized at a concentration of 0.05–0.1 g/ml in 100 mM Na-phosphate, 150 mM NaCl, 5% glycerol and 1 mM EDTA, pH 8.0. Detergents were added at different concentrations; the suspension was homogenized again, incubated for 30 min and centrifuged at $350\,000 \times g$ for 20 min. The fraction of protein solubilized was estimated by comparing the scanned integrated band intensities of the supernatant and SDS-solubilized pellet on Coomassie-stained SDS-PAGE. For purification, DDM, LDAO or *N*-laurylsarcosine was added to the resuspended membranes at 0.5–2% final concentration, the suspension was homogenized and incubated

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; rKch, recombinant Kch; sXXX kDa, ucXXX kDa, apparent molecular weight according to molecular sieve chromatography and analytical ultracentrifugation, respectively; DDM, dodecyl-β-D-maltopyranoside; LDAO, laurydimethylamine oxide

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for 1–2 h at 4°C. The supernatant from a centrifugation at 42 000 rpm in a Beckman 45 Ti rotor (about 140 000×*g*) for 90 min at 4°C was loaded onto a Ni-NTA column that had been equilibrated with buffer A containing 20 mM Na-phosphate, 20 mM imidazole, 50 mM NaCl, pH 8.0 and 0.04% DDM, 0.15% LDAO or *N*-laurylsarcosine. The column was washed with 500 mM NaCl in the same buffer and the protein was eluted with a linear gradient (0.02–1 M imidazole in 10 column volumes) in buffer A. rKch was concentrated with 100 kDa molecular cutoff Centriplus devices and the protein was run over a calibrated Superdex 200 high load 16/60 column in 20 mM Na-phosphate, pH 8.0 and appropriate detergent.

2.4. Analytical ultracentrifugation

Analytical sedimentation equilibrium runs of the purified, detergent-solubilized protein were carried out in a Beckman XL-A Analytical Ultracentrifuge at 3000 or 4000 rpm (600–1200×*g*) at 4°C. Before starting the analytical run, the protein samples were dialyzed against different buffer and detergent conditions and were spun at 350 000×*g* for 30 min to remove possible precipitate. During the analytical runs A_{280} (minus buffer absorbance) was monitored across the cell in radial direction. Assuming an ideal single particle solution the experimental data pairs were fitted to the equation

$$c(r) = c_F \exp\{\omega^2 M(1-v\rho)(r^2-r_F^2)/(2RT)\}$$

(where $c(r)$ is the concentration at radius r at equilibrium during centrifugation with angular velocity ω of the protein with mass M and specific volume v in a solution of mass density ρ at temperature T; r_F is the radius at the end of the cell closest to the rotation axis, $c_F = c(r_F)$ and R is the gas constant; v was assumed to be 0.77 ml/g and ρ to be 1.01 g/ml for a typical buffer used) with free c_F and M.

2.5. Circular dichroism (CD) spectroscopy

CD spectra of the detergent-solubilized, purified protein were recorded in 1 mm strain free quartz cuvettes in a Aviv CD Spectrometer model 62DS at 4°C from 300 nm to 190 nm (step size 1 nm, bandwidth 1.5 nm, 1 s averaging time). The CD signal Ψ (mdeg) was converted to mean residue ellipticity Θ (deg×cm^2 dmol^{-1}) by $\Theta = 100\,\Psi/(lcn)$ with the path length (cm) l, the protein concentration (mM) c and the number of residues $n = 417$. The protein concentration was determined according to the bicinchoninic assay (Pierce), the α-helix content was estimated according to Finkelstein et al. [7].

3. Results

3.1. Cloning and expression of rKch and cellular localization

pET28a(+) was chosen as it allows for the expression of the *kch* gene as a fusion protein with a His tag and T7 tag on the N-terminal end for effective purification and sensitive detection, respectively. The expression construct pET28a(+)-Kch was sequenced verifying the published DNA sequence [4]. For large-scale purification rKch was expressed in BL21(DE3) by induction with 0.1 mM IPTG and grown at 33°C in order to reduce formation of inclusion bodies. Besides its presence in the low centrifugation pellet (containing unbroken cells as well as inclusion bodies) the expected 50 kDa monomer and T7 antibody-stainable material was found in the high centrifugation pellet in such a high concentration that it could easily be detected on Coomassie-stained SDS-PAGE. No immunostaining occurred in samples of the high centrifugation supernatant. Sucrose density gradient centrifugations of crude membranes yielded two bands, the lower band (higher density) harboring most of the loaded material as determined by its optical density. This band has a mass density of 1.22–1.24 g/ml, while the weaker upper band has a mass density of about 1.18 g/ml. According to SDS-PAGE, over 85% of rKch is present in the high density band, whereas TLC with subsequent staining with ninhydrin (indicating amines) revealed a spot at the same position as *E. coli* lipids or dioleoyl phosphatidyl ethanolamine. Since phosphatidyl ethanolamine is the main constituent of the *E. coli* plasma membrane this suggests that rKch is located in the inner membrane.

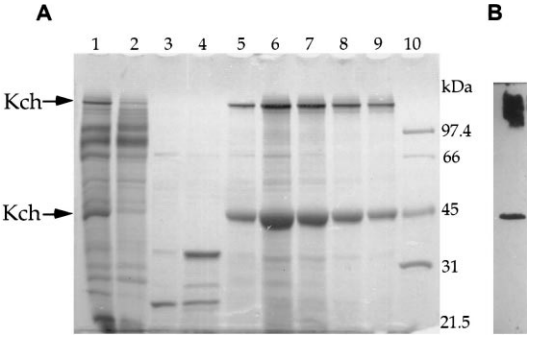

Fig. 1. Purification on Ni-NTA column. A: SDS-PAGE (10% gel, Coomassie stain). Lane 1: loaded onto column; lane 2: wash; lanes 3 and 4: NaCl wash; lanes 5–9: elution corresponding to 185, 250, 320, 380 and 450 mM imidazole; lane 10: low molecular weight marker. B: Western blot of the material loaded onto the column subsequently stained with T7 monoclonal antibody and horseradish peroxidase-conjugated mouse anti-goat antibody.

3.2. Solubilization and purification

The protein can be solubilized in detergents such as the anionic SDS, the zwitterionic zwittergents 3–12, 3–14, 3–16, *N*-laurylsarcosine, LDAO and the non-ionic DDM, tridecyl- and tetradecylmaltopyranoside (>50% rKch solubilized). Typical detergents used for the solubilization of eukaryotic potassium channels such as CHAPS, cholic acid or octylglucoside were found to have very poor solubilization capabilities (<5% rKch solubilized) even at high concentrations. rKch binds to the Ni-NTA column in phosphate buffer at pH 8.0 containing the detergents 0.02–0.04% DDM, 0.04% LDAO or 0.15–0.3% *N*-laurylsarcosine. The high salt wash step removes low molecular weight proteins (Fig. 1A, lane 4); increasing the imidazole concentration to 250–280 mM leads to the elution of rKch in a single peak (lanes 5–9). Peak integration showed that we obtain about 4–5 mg protein per liter culture from the Ni-NTA column when solubilized with DDM. On SDS-PAGE in Fig. 1A the eluted protein shows two sharp bands, one occurring at about 50 kDa, the expected monomeric size, the other at over 400 kDa, referred to as the monomeric and oligomeric bands, respectively. In order to determine if the two SDS-PAGE bands are of rKch, we subjected both bands to peptide fingerprinting and microsequencing. The analysis gave very similar peptide maps for the two bands, and all peptide samples that were sequenced were comprised of peptides from the deduced rKch amino acid sequence. Other evidence that both bands on SDS-PAGE contain rKch comes from the fact that both bands are recognized by the T7 monoclonal antibody (Fig. 1B), although there appears to be additional staining of aggregated material in the loading pocket.

3.3. Size determinations

Concentration of rKch in DDM, LDAO and *N*-laurylsarcosine without detectable protein loss is possible using 100 kDa molecular cut-off membranes suggesting that rKch forms oligomers. Fig. 2A shows elution profiles of molecular sieve chromatography (Superdex 200) runs performed with different running buffers and detergents. These profiles can be obtained with and without the additional concentration step (protein

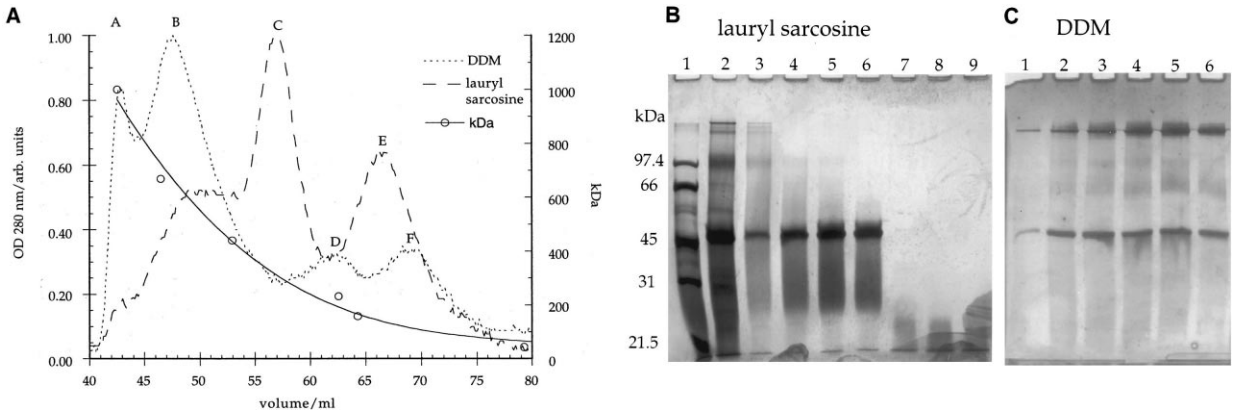

Fig. 2. Ni-NTA-purified rKch chromatographed on Superdex 200 16/60. A: Profiles: DDM run: 20 mM Na-phosphate, pH 8.0, 140 mM KCl, 0.02% DDM or 0.04% LDAO; laurylsarcosine run: 10 mM Tris, pH 8.0, 0.3% *N*-laurylsarcosine (solubilized in 2%). B: SDS-PAGE of laurylsarcosine run. Lane 1: low molecular weight marker; lane 2: loaded protein; lanes 3–9: 48–50, 54–56, 56–58, 58–60, 64–66, 66–68, 68–70 ml. C: SDS-PAGE of DDM run (very similar to LDAO runs): lanes 1–6: 40–42, 42–44, 44–46, 46–48, 48–50, 50–52 ml.

concentrations between 3.5 and 0.2 mg/ml) after Ni-NTA chromatography. rKch behaves very similarly in DDM and LDAO where the peaks A, B, D and F were observed (see Fig. 2A, DDM run). Peak A (> s650 kDa), which runs at the exclusion volume of the column, contains partly aggregated material containing rKch when analyzed on SDS-PAGE. Peak B (s570–630 kDa) is the major peak in DDM or LDAO, and contains monomeric and oligomeric bands of rKch on SDS-PAGE with the same intensity ratio as the ones already obtained in the elution of the Ni-NTA column (compare Figs. 1A and 2C). The remaining peaks D (s200–230 kDa) and F (s110–120 kDa) contain lipid-detergent micelles. When the protein is solubilized and purified in *N*-laurylsarcosine a very different molecular mass pattern evolves (see Fig. 2A, laurylsarcosine run). As can be seen in Fig. 2B, lanes 4–6, peak C (s300–340 kDa) runs on silver-stained SDS-PAGE as a monomeric rKch band without any residual oligomeric rKch, whereas peak E (lanes 7–9) does not contain protein but stainable material in the running front of the gel corresponding to lipid-detergent micelles.

To independently determine the molecular size of rKch in solution we utilized sedimentation equilibrium centrifugations. Several samples eluted from the Ni-NTA column in DDM and dialyzed against different buffers, and peaks A and B from the molecular sieve chromatography run were analyzed. Samples in DDM from the Ni-NTA column prior to molecular sieve chromatography showed molecular masses of uc270 (Fig. 3A). Other samples from the Ni-NTA column in the detergents 0.1% decylmaltopyranoside or 0.04% LDAO and *N*-laurylsarcosine gave similar masses in the range of uc260–uc300 kDa. In contrast, we found for peak A (Fig. 3B) a homogeneous solution with particles of mass uc790 kDa. Peak B contained different particles with an average mass of uc580 kDa. This indicates that at least in the detergents DDM or LDAO the molecular sieve run using the Superdex 200 column resulted in the aggregation of particles of about uc280 kDa to form a mixture of oligomers having a maximum weight of about uc790 kDa. The only detergent we have found where this appears not to happen is *N*-laurylsarcosine.

3.4. CD spectra

CD spectra of rKch purified using the Ni column were recorded in 10 mM Na-phosphate, 0.04% DDM, pH 8.0 (Fig. 4). The protein shows spectra typical for α-helical pro-

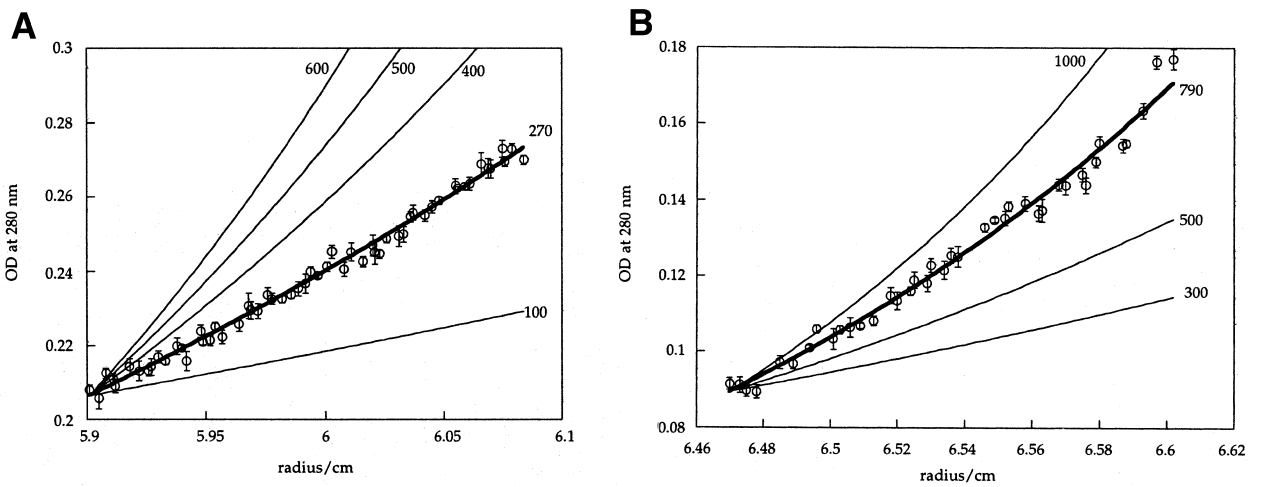

Fig. 3. Absorption profiles from equilibrium analytical centrifugation of purified rKch. Measured data (open circles with error bars) as well as best fits (lines) are included. The numbers are molecular masses in kDa deduced from the fits. For comparison, curves corresponding to some other molecular weights are included. A: From Ni-NTA column in 0.03% DDM; B: peak A of the DDM run in Fig. 2A.

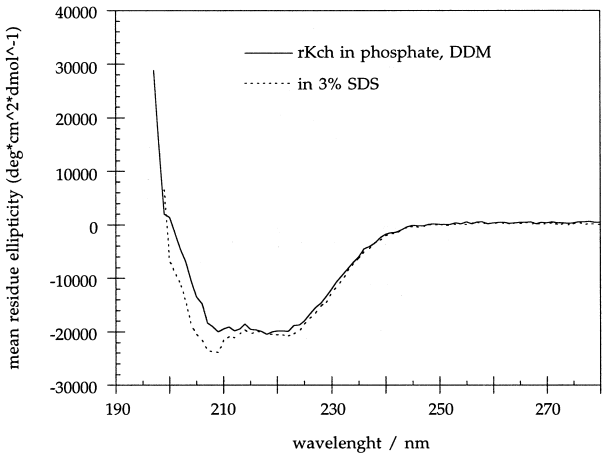

Fig. 4. CD spectra of rKch. Purified rKch in DDM and phosphate buffer and upon subsequent addition of SDS up to 3%.

tein having minima at 208 nm and 222 nm that did not change significantly upon addition of SDS up to 3%. Using the ellipticity values at 222 nm the α-helix content was estimated to about 56%. The smaller value at 208 nm of the 3% SDS sample might be due to dissolving partly aggregated protein. Similar spectra were obtained in Tris buffer at the same pH.

4. Discussion

We report here the recombinant expression and characterization of the recently found gene *kch* from *E. coli* that shows similarities to eukaryotic membrane-spanning potassium channels [4,5]. The membrane fraction containing most rKch consists mainly of phosphatidyl ethanolamine and has a mass density of about 1.23 g/ml. The inner membrane of the wild type cell has a lower density of about 1.18 g/ml indicating that the protein/lipid ratio in the inner membrane is significantly increased upon overexpression of Kch in *E. coli*. We were able to solubilize rKch with detergents possessing an alkyl chain at least 12 carbon atoms long. The protein eluted from the Ni-NTA column is in an oligomeric form and has a molecular mass of about uc270 kDa in DDM, LDAO, DM and *N*-laurylsarcosine according to analytical ultracentrifugations. Considering the additional mass of the detergent shell, the uc270 kDa particles might represent tetramers of the 50 kDa monomer. Voltage-gated potassium channels have been shown to form homotetramers [2,8–10] and assembly domains have been identified at the N-termini [11,12]. Since Kch has a very short N-terminal segment, different regions than in the case of eukaryotic potassium channels might be involved in the oligomerization process. Based on molecular sieve chromatography, the particle size of rKch solubilized in N-laurylsarcosine is about 320 kDa; considering that the detergent micelle alone is about 30 kDa, this indicates that the solubilized rKch is maintained as tetramers. These particles dissociate on SDS-PAGE. In contrast, molecular sieve chromatography of rKch solubilized in DDM or LDAO results in the formation of even larger particles of about s580–620 kDa and over s650 kDa (uc580 kDa and uc790 kDa, respectively by analytical ultracentrifugation) representing possibly dodecamers of rKch stable on SDS-PAGE. The presence of smaller molecular weight particles (tetramers) before the size exclusion run and elution of lipid-detergent micelles from this column in DDM and LDAO suggests that delipidation might cause aggregation into higher oligomers in these detergents. The observed differences in mass determinations between the two techniques might be due to the sensitivity of the calculated apparent mass on the choice of parameter values. If the actual specific protein volume v was in fact 5% larger (smaller) than the assumed value of 0.77 ml/g, the determined protein mass from the absorption curves would be 1.2 (0.88) times the values given above. Therefore we regard the mass determinations not as absolute values but as estimations, especially to compare the oligomerization behavior of Kch in different buffers and detergents. Taken together, these data suggest that *N*-laurylsarcosine is more effective than other tested detergents in preventing aggregation of rKch tetramers into higher oligomers. CD spectroscopic measurements suggest that rKch is highly α-helical in solubilized oligomeric form which is expected for all voltage-gated potassium channels. The secondary structure seems to prevail also after addition of SDS which agrees with the behavior of the protein in SDS-PAGE, where we observe partial preservation of defined oligomers. Stability of tetramers in SDS-PAGE is known for the prokaryotic inwardly rectifying potassium channel *KcsA* [13,14].

In summary, these results indicate that Kch shares similarities with eukaryotic potassium channels not only in primary structure but also in biochemical properties. On the other hand, there are striking differences at least in primary sequence that are expected to be closely related to putative channel function. The S4 region in Kch does not show the regular spacing of basic and leucine residues characteristic of known voltage-gated channels and shown to be involved in voltage-dependent activation in these channels [1,3]. As for the recently found potassium channel in yeast [15,16] which does not contain a positively charged S4 segment, involvement of external factors could also be required for the activation of a putative Kch channel. The N-terminal region being very short for the *E. coli* protein constitutes another difference between Kch and eukaryotic potassium channels where it acts as a potential inactivation motif [17,18].

The presented work lays the foundation for further studies of Kch. Of particular interest will be the determination of the actual physiological function of this protein. The possibility to obtain milligram amounts is attractive to structural biologists, so that crystallization trials in two and three dimensions can be started for subsequent molecular structure determination.

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References

[1] Hille, B. (1992) Sinauer Press, Sunderland, MA.
[2] MacKinnon, R. (1991) Nature 350, 232–235.
[3] Jan, L.Y. and Jan, Y.N. (1997) Annu. Rev. Neurosci. 20, 91–123.
[4] Milkman, R. (1994) Proc. Natl. Acad. Sci. USA 91, 3510–3514.
[5] Johansson, M. and von Heijne, G. (1996) J. Biol. Chem. 271, 25912–25915.

[6] Osborn, M.J. and Munson, R. (1974) Methods Enzymol. 31, 642–653.
[7] Finkelstein, A.V., Ptitsyn, O.B. and Kozitsyn, S.A. (1977) Biopolymers 16, 497–524.
[8] Liman, E.R., Tytgat, J. and Hess, P. (1992) Neuron 9, 861–871.
[9] Schulteis, C.T., Nagaya, N. and Papazian, D.M. (1996) Biochemistry 35, 12133–12140.
[10] Li, M., Unwin, N., Stauffer, K.A., Jan, Y.N. and Jan, L.Y. (1994) Curr. Biol. 4, 110–115.
[11] Shen, N.V. and Pfaffinger, P.J. (1995) Neuron 14, 625–633.
[12] Xu, J., Yu, W., Jan, Y.N., Jan, L.Y. and Li, M. (1995) J. Biol. Chem. 270, 24761–24768.
[13] Cortes, D.M. and Perozo, E. (1997) Biochemistry 36, 10343–10352.
[14] Heginbotham, L., Odessey, E. and Miller, C. (1997) Biochemistry 36, 10335–10342.
[15] Zhou, X.L., Vaillant, B., Loukin, S.H., Kung, C. and Saimi, Y. (1995) FEBS Lett. 373, 170–176.
[16] Ketchum, K.A., Joiner, W.J., Sellers, A.J., Kaczmarek, L.K. and Goldstein, S.A. (1995) Nature 376, 690–695.
[17] Hoshi, T., Zagotta, W.N. and Aldrich, R.W. (1990) Science 250, 533–538.
[18] Zagotta, W.N., Hoshi, T. and Aldrich, R.W. (1990) Science 250, 568–571.